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(54) Title: METHODS FOR STIMULATING THE REGENERATION OF INNER EAR CELLS

(57) Abstract

In one aspect, the present invention provides methods for stimulating the regeneration of inner ear cells comprising introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of an inner ear cell, into at least one inner ear cell under conditions that enable expression of the transcription factor in the inner ear cell. Any type of inner ear cell can be treated in accordance with the present invention, but the presently preferred cells treated in accordance with the present invention are inner ear supporting cells. The presently preferred gene delivery method is lipofection. In another aspect, the present invention provides methods for ameliorating the symptoms of an inner ear disease comprising introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of an inner ear cell, into a portion of the inner ear under conditions that enable the uptake of the nucleic acid molecule by inner ear cells and expression of the transcription factor within the inner ear cells. In yet another aspect, the present invention provides genetically-transformed, regenerated inner ear cells, preferably supporting cells or sensory hair cells, including a transgene encoding an inner ear cell transcript or factor.

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METHODS FOR STIMULATING THE REGENERATION OF INNER EAR CELLS

Field of the Invention

The invention relates to the promotion of inner ear sensory cell regeneration by introducing transcription factor genes such as the transcription factor POU4F3.

Background of the Invention

Sensorineuronal hearing loss (SNHL), also called "nerve deafness," is a significant communication problem that affects tens of millions of people in the U.S. alone. Loss of the inner ear sensory cell (i.e., hair cells) is thought to be a major cause of this deficit. SNHL can be caused by a multitude of events including age-related loss (presbycusis), noise exposure, drug exposure (e.g., antibiotics and anti-cancer therapeutics), infections, genetic mutations (syndromic and non-syndromic) and autoimmune disease. Unfortunately, loss of auditory hair cells is permanent with no regeneration having been observed in any mammalian species.

Currently, the treatment for acquired SNHL involves the use of external [illegible] and cochlear implants. Both devices have rather limited therapeutic

Recent observations suggest that cells within the organ of Corti are capable of transiently adopting an immature hair cell-like phenotype following a lesion of the hair cells by an aminoglycoside antibiotic (Lenoir, M. and Vago, P., *C.R. Acad. Sci. Pari, Life Sci.*, **319:**269-276 (1996); Lenoir, M. and Vago, P., *Int. J. Devl. Neuroscience*, **15(4/5):**487-495 (1997)). This phenomenon is termed "neodifferentiation." In response to amikacin, cells in the apical turn of the two to three week-old cochlea began to display stereocilia-like processes from their luminal surfaces at the cessation of amikacin treatment. Unfortunately, this phenotype was not maintained at longer recovery time points, which showed only the presence of a characteristic supporting-cell phalangeal scar (Forge, A., *Hear. Res.*, **19:**171-182 (1985); Raphael, Y., and Altschuler, R. "Reorganization of cytoskeletal and junctional proteins during cochlear hair cell regeneration" *Cell Motil. Cytoskeleton*, **18:**215-217 (1991a); Raphael, Y., and Altschuler, R., *Hear. Res.*, **51:**173-184 (1991b); Raphael, Y., and Altschuler, R. "Early microfilament reorganization in injured auditory epithelia" *Exp. Neurol.*, **115:**32-36 (1992)).

As a model system for studying the effects of hair cell death, efforts have been made to develop methods for selectively killing hair cells in mice by administering aminoglycoside antibiotics ("lesioning"). However, lesioning apical turn hair cells with aminoglycosides in previously reported organ of Corti culture systems remains difficult (Richardson, G.P., and Russell, I.J., *Hear. Res.*, **53:**293-311 (1991); Chardin, S., and Romand, R., *Science*, **267:**707-709 (1995)). At the time of explant in neonatal cultures (day of birth to postnatal day three (P3)), apical turn hair cells are highly resistant to aminoglycoside-induced death and therefore such cultures are not suitable to the study of hair cell regeneration. Recently, a method for lesioning the apical turn of the older neonatal organ of Corti, i.e., P7-14, was developed (Kil, J. et al., *ARO Abstracts*, **21:**672 (1998); this publication is hereby incorporated by reference in its entirety). In P7-14 mice, apical turn hair cells are more susceptible to aminoglycoside ototoxicity than younger embryos, and can be effectively lesioned. Reasons for this increased susceptibility remain unknown, but may be due to the onset of auditory function at this time in development.

To date, both *in vivo* and *in vitro* attempts at inducing auditory hair cell regeneration in the postnatal period have revolved around the addition of exogenous

appropriate receptor expression by the remaining supporting cells. Another reason may be a lack of the appropriate intracellular process(s) that would allow a non-sensory supporting cell to develop into a sensory hair cell. In birds, where auditory hair cells do regenerate after lesioning, the neighboring supporting cells give rise to new hair cells (Corwin, J.T., and Cotanche, D., *Science*, **240**:1772-74 (1988); Ryals, B.M., and Rubel, E.W., *Science*, **240**:1774-76 (1988)).

Recent discoveries indicate that hair cell development is dependent upon the function of a POU domain transcription factor called POU4F3. Brn 3c (also called "3.1") (the mouse homolog of human POU4F3) is expressed in limited regions of the brain, retina, dorsal root and trigeminal ganglia, and in inner ear sensory hair cells (Xiang, M. et al., *J. Neuroscience*, **15(7)**:4762-4785 (1995)). Homozygous deletion in mice of the Brn 3c gene results in the failure of inner ear hair cells to develop and differentiate in the postnatal period of mice (Erkman, L., et al., *Nature*, **381**:603-606 (1996); Xiang, M et al., *Proc. Natl. Acad. Sci. USA*, **94**:9445-9450 (1997)). Mice with this deletion have permanent deficits in hearing and balance, while heterozygous mice have no obvious deficits as compared with wild-type mice. Interestingly, a form of adult-onset SNHL was identified in an Israeli family with a mutation in one allele of POU4F3 that develops SNHL from the age of 18 to 30 (Vahava, O. et al., *Science*, **279**:1950-1954 (1998)).

A need exists for methods of stimulating the regeneration of inner ear cells. In particular, a need exists for methods for regenerating inner ear, sensory hair cells, in subjects suffering from sensory hair cell death or degeneration, thereby ameliorating the symptoms of inner ear diseases caused by the deterioration and/or death of inner ear sensory hair cells.

Summary of the Invention

In one aspect, the present invention provides methods for stimulating the regeneration of inner ear cells comprising introducing a nucleic acid molecule (such as the nucleic acid molecule set forth in SEQ ID NO:1), that encodes an inner ear cell transcription factor capable of stimulating the regeneration of inner ear cells, into at least one inner ear cell under conditions that enable expression of the transcription factor in the inner ear cell. Presently preferred, inner ear cell transcription factors are capable of stimulating the regeneration of sensory hair cells from non-sensory cells

[illegible] preferably at least 50% homologous most

[illegible] nucleic acid sequence set forth in SEQ ID NO:1. [illegible] presently preferred nucleic acid

molecules encoding an inner ear cell transcription factor encode a POU4F3 homologue and hybridize to the nucleic acid molecule set forth in SEQ ID NO:1 (or to its complementary sequence) under stringent conditions. Any type of inner ear cell can be treated in accordance with the present invention, but the presently preferred cells treated in accordance with the present invention are inner ear supporting cells. In a presently most preferred embodiment of this aspect of the invention, inner ear sensory hair cells are regenerated from inner ear supporting cells. In the practice of the present invention, any art-recognized gene delivery method can be used to introduce a nucleic acid molecule, encoding an inner ear cell transcription factor, into inner ear cells for expression therein. The presently preferred gene delivery method is lipofection.

In another aspect, the present invention provides methods for ameliorating the symptoms of an inner ear disease. In a presently preferred embodiment, the methods for ameliorating the symptoms of an inner ear disease comprise introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of inner ear cells, into a portion of the inner ear (preferably the cochlea, semicircular canals and/or otolithic organs) under conditions that enable the uptake of the nucleic acid molecule by inner ear cells and expression of the transcription factor within the inner ear cells. Presently preferred inner ear cells suitable for treatment in accordance with this aspect of the present invention are inner ear supporting cells. In a presently most preferred embodiment of this aspect of the invention, inner ear sensory hair cells are regenerated from inner ear supporting cells. A nucleic acid molecule that encodes an inner ear cell transcription factor can be introduced into a portion of the inner ear by any art-recognized means, such as by injection. A presently preferred method for introducing a nucleic acid molecule, that encodes an inner ear cell transcription factor, into the cochlea is by cochleostomy.

In another aspect, the present invention provides genetically-transformed inner ear cells, preferably supporting cells or sensory hair cells, including a transgene encoding an inner ear cell transcription factor.

Brief Description of the Drawings

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by

[illegible] FIGURE [illegible]

Detailed Description of the Preferred Embodiment

As used herein, the terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids or their residues. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan
Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

As used herein, the term "nucleotide" means a monomeric unit of DNA or RNA containing a sugar moiety (pentose), a phosphate and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide with the four bases of DNA being adenine ("A"), guanine ("G"), cytosine ("C") and thymine ("T"). Inosine ("I") is a synthetic base that can be used to substitute for any of the four, naturally-occurring bases (A, C, G or T). The four RNA bases are A,G,C and uracil ("U"). The nucleotide sequences described herein comprise a linear array of nucleotides connected by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

"Oligonucleotide" refers to short length single or double stranded sequences of deoxyribonucleotides linked via phosphodiester bonds. The oligonucleotides are chemically synthesized by known methods and purified, for example, on polyacrylamide gels.

As used herein, the term "supporting cells", or "inner ear supporting cells", and grammatical equivalents thereof, means cells that physically support, or provide nutrients to, sensory inner ear cells, such as the sensory hair cells of the Organ of Corti. By way of non-limiting example, supporting cells include border cells , inner

As used herein, the term "inner ear cell transcription factor", and grammatical equivalents thereof, refers to transcription factors that are normally expressed in at least one type of developing and/or mature inner ear cell, and have the ability to stimulate regeneration of inner ear cells, preferably stimulate the regeneration of inner ear sensory hair cells from supporting cells, when utilized in the practice of the methods of the present invention. Typically, inner ear cell transcription factors useful in the practice of the present invention are required for the normal development, and/or for the normal functioning, of the inner ear cell type(s) in which they are normally expressed.

Representative examples of inner ear cell transcription factors useful in the practice of the present invention include POU4F1 (Collum, R.G. et al., Nucleic Acids Research **20(18)**:4919-4925 (1992)), POU4F2 (Xiang et al., Neuron **11**:689-701 (1993)), POU4F3 (Vahava, O., Science **279(5358)**:1950-1954 (1998), Brn3a (also known as Brn3.0), Brn3b (also known as Brn3.2) and Brn3c (also known as Brn3.1) as disclosed in Gerrero et al., Proc. Nat'l. Acad. Sci. (U.S.A.) **90(22)**:10841-10845 (1993), Xiang, M. et al., Proc. Nat'l. Acad. Sci. (U.S.A.) **93(21)**:11950-11955 (1996), Xiang, M. et al., J. Neurosci. **15(7Part 1)**:4762-4785 (1995), Erkman, L. et al., Nature **381(6583)**:603-606 (1996), Xiang, M. et al., Proc. Nat'l. Acad. Sci. (U.S.A.) **94(17)**:9445-9450 (1997), each of which publications is incorporated herein by reference. Presently preferred inner ear cell transcription factors possess at least one homeodomain and/or at least one POU-specific domain, and have a molecular weight in the range of from about 33 kDa to about 37 kDa.

As used herein, the term "homeodomain" means an amino acid sequence that is at least 50% homologous, more preferably at least 75% homologous, most preferably at least 90% homologous to the homeodomain amino acid sequence set forth in SEQ ID NO:3. In this context, amino acid sequence homology (also referred to as amino acid sequence identity) is defined as the percentage of amino acid residues in the amino acid sequence set forth in SEQ ID NO:3 that are identical with part or all of a candidate protein sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology (identity), and not considering any conservative substitutions as part of the sequence homology. Neither N- or C-terminal extensions nor insertions shall be construed as reducing homology. No

As used herein, the term "POU-specific domain" means an amino acid sequence that is at least 50% homologous, more preferably at least 75% homologous, most preferably at least 90% homologous to the POU-specific domain amino acid sequence set forth in SEQ ID NO:4. In this context, amino acid sequence homology (also referred to as amino acid sequence identity) is defined as the percentage of amino acid residues in the amino acid sequence set forth in SEQ ID NO:4 that are identical with part or all of a candidate protein sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology (identity), and not considering any conservative substitutions as part of the sequence homology. Neither N- or C- terminal extensions nor insertions shall be construed as reducing homology. No weight is given to the number or length of gaps introduced, if necessary, to achieve the maximum percent homology (identity).

Presently more preferred inner ear cell transcription factors are POU4F3 transcription factor homologues (hereinafter referred to as POU4F3 homologues). POU4F3 homologues useful in the practice of the present invention are capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells) and are at least 25% homologous, preferably at least 50% homologous, more preferably at least 75% homologous, most preferably at least 90% homologous to the POU4F3 transcription factor having the amino acid sequence set forth in SEQ ID NO:2. In this context, amino acid sequence homology (also referred to as amino acid sequence identity) is defined as the percentage of amino acid residues in the POU4F3 transcription factor having the amino acid sequence set forth in SEQ ID NO:2 that are identical with part or all of a candidate protein sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology (identity), and not considering any conservative substitutions as part of the sequence homology. Neither N- or C- terminal extensions nor insertions shall be construed as reducing homology. No weight is given to the number or length of gaps introduced, if necessary, to achieve the maximum percent homology (identity). As used herein, the term "POU4F3 homologues" includes the POU4F3 protein having the amino acid sequence set forth in SEQ ID NO:2, which is the presently most preferred inner ear cell transcription factor useful in the practice of the present invention. Representative [illegible] of the present invention [illegible] herein by reference.

As used herein, the term "stimulate regeneration of an inner ear cell", and grammatical equivalents, means to stimulate the complete or partial regeneration of an inner ear cell. For example, in a presently preferred embodiment of the invention, inner ear sensory hair cells are regenerated from inner ear supporting cells. Preferably, fully functional inner ear cells are regenerated.

The terms "alteration", "amino acid sequence alteration", "variant" and "amino acid sequence variant" refer to protein molecules with some differences in their amino acid sequences as compared to the corresponding, native, *i.e.*, naturally-occurring, proteins. Ordinarily, the variants will possess at least about 70% homology with the corresponding native protein, and preferably, they will be at least about 80% homologous with the corresponding, native protein. The amino acid sequence variants of the transcription factors useful in the practice of the present invention possess substitutions, deletions, and/or insertions at certain positions. Sequence variants of inner ear cell transcription factors may, for example, be more effective at stimulating regeneration of inner ear cells when used in accordance with the present invention.

Substitutional protein variants are those that have at least one amino acid residue in the native protein sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule. Substantial changes in the activity of the proteins useful in the practice of the present invention may be obtained by substituting an amino acid with a side chain that is significantly different in charge and/or structure from that of the native amino acid. This type of substitution would be expected to affect the structure of the polypeptide backbone and/or the charge or hydrophobicity of the molecule in the area of the substitution.

Moderate changes in the activity of the transcription factor molecules useful in the present invention would be expected by substituting an amino acid with a side chain that is similar in charge and/or structure to that of the native molecule. This type of substitution, referred to as a conservative substitution, would not be expected to substantially alter either the structure of the polypeptide backbone or the charge or hydrophobicity of the molecule in the area of the substitution.

α-carboxy or α-amino functional group of the amino acid. The insertion may be one or more amino acids. Ordinarily, the insertion will consist of one or two conservative amino acids. Amino acids similar in charge and/or structure to the amino acids adjacent to the site of insertion are defined as conservative. Insertional protein variants also include insertion of an amino acid with a charge and/or structure that is substantially different from the amino acids adjacent to the site of insertion.

Deletional variants are those where one or more amino acids in the native protein molecules have been removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the protein molecule.

The terms "DNA sequence encoding", "DNA encoding" "nucleic acid molecule encoding" and "nucleic acid encoding", and grammatical equivalents thereof, refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the translated polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The terms "replicable vector" "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it another piece of DNA (the insert DNA) such as, but not limited to, a cDNA molecule. The vector is used to transport the insert DNA into a suitable host cell. The insert DNA may be derived from the host cell, or may be derived from a different cell or organism. Once in the host cell, the vector can replicate independently of or coincidental with the host chromosomal DNA, and several copies of the vector and its inserted DNA may be generated. The terms "replicable expression vector" and "expression vector" refer exclusively to vectors that contain the necessary elements that permit the expression of a polypeptide encoded by the insert DNA. Many molecules of the polypeptide encoded by the insert DNA can thus be rapidly synthesized.

The terms "transformed host cell," "transformed" and "transformation" refer to the introduction of DNA into a cell. The cell is termed a "host cell", and it may be a prokaryotic or a eukaryotic cell. Typical prokaryotic host cells include various strains of *E. coli*. Typical eukaryotic host cells are insect cells or animal cells, such as inner ear supporting cells. The introduced DNA is usually in the form of a vector

may be a hybrid DNA sequence, containing some foreign DNA and some DNA derived from the host species.

In one aspect, the present invention provides methods for stimulating the regeneration of inner ear cells comprising introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells), into at least one inner ear cell under conditions that enable expression of the transcription factor in the inner ear cell.

Any type of inner ear cell can be treated in accordance with the present invention, but the presently preferred cells treated in accordance with the present invention are supporting cells. Supporting cells underlie and physically support sensory hair cells within the inner ear. The anatomy of the inner ear is well known to those of ordinary skill in the art (*see, e.g.*, *Gray's Anatomy*, Revised American Edition (1977), pages 859-867, incorporated herein by reference). In brief, the inner ear includes three sensory portions: the cochlea, which senses sound; the semicircular canals, which sense angular acceleration; and the otolithic organs, which sense linear acceleration. In each of these sensory portions, specialized sensory hair cells are arrayed upon one or more layers of inner ear supporting cells. In operation, the sensory hair cells are physically deflected in response to sound or motion, and their deflection is transmitted to nerves which send nerve impulses to the brain for processing and interpretation. Some types of hearing disorders result from the death or deterioration of sensory hair cells.

In particular, the cochlea includes the Organ of Corti which is primarily responsible for sensing sound. As shown in the FIGURE, the Organ of Corti 10 includes a basilar membrane 12 upon which are located a variety of supporting cells 14, including border cells 16, inner pillar cells 18, outer pillar cells 20, inner phalangeal cells 22, Dieter's cells 24 and Hensen's cells 26. Supporting cells 14 support inner hair cells 28 and outer hair cells 30. Tectorial membrane 32 is disposed above inner hair cells 28 and outer hair cells 30. In the presently most preferred embodiment, the present invention is adapted to stimulate regeneration of sensory hair cells 28 and 30 from underlying supporting cells 14.

Preferably, the nucleic acid molecules that encode inner ear cell transcription

the present invention are cDNA molecules that encode an inner ear cell transcription

factor, capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells), that possesses at least one homeodomain and/or at least one POU-specific domain, and has a molecular weight in the range of from about 33 kDa to about 37 kDa.. Presently more preferred nucleic acid molecules useful in the practice of the present invention encode a POU4F3 homologue capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells). A presently most preferred nucleic acid molecule that encodes a POU4F3 homologue is the nucleic acid molecule having the nucleic acid sequence set forth in SEQ ID NO:1.

Additional nucleic acid molecules encoding transcription factors useful in the practice of the present invention can be isolated by using a variety of cloning techniques known to those of ordinary skill in the art. For example, cloned POU4F3 homologues cDNAs or genes, or fragments thereof, can be used as hybridization probes utilizing, for example, the technique of hybridizing radiolabelled nucleic acid probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes as set forth at pages 9.52 to 9.55 of *Molecular Cloning, A Laboratory Manual* (2nd edition), J. Sambrook, E.F. Fritsch and T. Maniatis eds., the cited pages of which are incorporated herein by reference. Presently preferred hybridization probes for identifying additional nucleic acid molecules encoding POU4F3 homologues are fragments, of at least 15 nucleotides in length, of the cDNA molecule (or its complementary sequence) having the nucleic acid sequence set forth in SEQ ID NO:1, although the complete cDNA molecule having the nucleic acid sequence set forth in SEQ ID NO:1 is also useful as a hybridization probe for identifying additional nucleic acid molecules encoding POU4F3 homologue. A presently most preferred hybridization probe for identifying additional nucleic acid molecules encoding POU4F3 homologues is the oligonucleotide having the nucleic acid sequence TAG AAG TGC AGG GCA CGC TGC TCA TGG TAT G (SEQ ID NO:5)

Exemplary high stringency hybridization and wash conditions useful for identifying (by Southern blotting) additional nucleic acid molecules encoding POU4F3 homologues are: hybridization at 68°C in 0.25 M Na_2HPO_4 buffer (pH 7.2) containing 1 mM Na_2EDTA, 20% sodium dodecyl sulfate; washing (three washes of

Exemplary moderate stringency hybridization and wash conditions useful for identifying (by Southern blotting) additional nucleic acid molecules encoding POU4F3 homologues are: hybridization at 45°C in 0.25 M Na_2HPO_4 buffer (pH 7.2) containing 1 mM Na_2EDTA, 20% sodium dodecyl sulfate; washing is conducted in 5X SSC, containing 0.1% (w/v) sodium dodecyl sulfate, at 55°C to 65°C. The abbreviation "SSC" refers to a buffer used in nucleic acid hybridization solutions. One liter of the 20X (twenty times concentrate) stock SSC buffer solution (pH 7.0) contains 175.3 g sodium chloride and 88.2 g sodium citrate.

Additional nucleic acid molecules encoding transcription factors useful in the practice of the present invention can also be isolated, for example, by the polymerase chain reaction (PCR) described in *The Polymerase Chain Reaction* (K.B. Mullis, F. Ferre, R.A. Gibbs, eds), Birkhauser Boston (1994), incorporated herein by reference. Preferably, PCR primers will be designed against conserved amino acid sequence motifs found in most or all of the known, target protein sequences. Examples of conserved amino acid sequence motifs against which PCR primers can be designed for cloning additional POU4F3 homologues are the POU-specific domain having the amino acid sequence set forth in SEQ ID NO:4, and the homeodomain having the amino acid sequence set forth in SEQ ID NO:3.

Further, additional nucleic acid molecules encoding transcription factors useful in the practice of the present invention can also be isolated, for example, by utilizing antibodies that recognize transcription factor proteins. Methods for preparing monoclonal and polyclonal antibodies are well known to those of ordinary skill in the art and are set forth, for example, in chapters five and six of *Antibodies A Laboratory Manual*, E. Harlow and D. Lane, Cold Spring Harbor Laboratory (1988), the cited chapters of which are incorporated herein by reference. By way of non-limiting example, antibodies were successfully raised against a fusion protein constructed from the C-terminal end of Brn3. Xiang M. et al., J. Neuroscience **15(7)**:4762-4785 (1995) and Xiang M. et al., P.N.A.S. (U.S.A.) **94**:9445-9450 (1997), incorporated herein by reference.

By way of non-limiting example, a cDNA expression library can be screened using anti-POU4F3 homologue antibodies in order to identify one or more clones that encode a POU4F3 homologue protein. DNA expression library technology is well

2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, the cited chapter of which is incorporated herein by reference.

Sequence variants, produced by deletions, substitutions, mutations and/or insertions, of the transcription factors useful in the practice of the present invention can also be used in the methods of the present invention. The amino acid sequence variants of the transcription factors useful in the practice of the present invention may be constructed by mutating the DNA sequences that encode the wild-type transcription factor proteins, such as by using techniques commonly referred to as site-directed mutagenesis. Nucleic acid molecules encoding the transcription factors useful in the practice of the present invention can be mutated by a variety of PCR techniques well known to one of ordinary skill in the art. (See, for example, the following publications, the cited portions of which are incorporated by reference herein: "PCR Strategies", M.A. Innis, D.H. Gelfand and J.J. Sninsky, eds., 1995, Academic Press, San Diego, CA (Chapter 14); "PCR Protocols: A Guide to Methods and Applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds., Academic Press, NY (1990)).

By way of non-limiting example, the two primer system utilized in the Transformer Site-Directed Mutagenesis kit from Clontech, may be employed for introducing site-directed mutants into nucleic acid molecules encoding transcription factors useful in the practice of the present invention. Following denaturation of the target plasmid in this system, two primers are simultaneously annealed to the plasmid; one of these primers contains the desired site-directed mutation, the other contains a mutation at another point in the plasmid resulting in elimination of a restriction site. Second strand synthesis is then carried out, tightly linking these two mutations, and the resulting plasmids are transformed into a *mutS* strain of *E. coli*. Plasmid DNA is isolated from the transformed bacteria, restricted with the relevant restriction enzyme (thereby linearizing the unmutated plasmids), and then retransformed into *E. coli*. This system allows for generation of mutations directly in an expression plasmid, without the necessity of subcloning or generation of single-stranded phagemids. The tight linkage of the two mutations and the subsequent linearization of unmutated plasmids results in high mutation efficiency and allows minimal screening. Following synthesis of the initial restriction site primer, this method requires the use of only one new primer type per mutation site. Rather than prepare each positional mutant

[illegible] desired mutations at a given site simultaneously

Transformants can be screened by sequencing the plasmid DNA through the mutagenized region to identify and sort mutant clones. Each mutant DNA can then be fully sequenced or restricted and analyzed by electrophoresis on Mutation Detection Enhancement gel (J.T. Baker) to confirm that no other alterations in the sequence have occurred (by band shift comparison to the unmutagenized control).

Again, by way of non-limiting example, the two primer system utilized in the QuikChange™ Site-Directed Mutagenesis kit from Stratagene (La Jolla, California), may be employed for introducing site-directed mutants into nucleic acid molecules encoding transcription factors useful in the practice of the present invention. Double-stranded plasmid DNA, containing the insert bearing the target mutation site, is denatured and mixed with two oligonucleotides complementary to each of the strands of the plasmid DNA at the target mutation site. The annealed oligonucleotide primers are extended using *Pfu* DNA polymerase, thereby generating a mutated plasmid containing staggered nicks. After temperature cycling, the unmutated, parental DNA template is digested with restriction enzyme *DpnI* which cleaves methylated or hemimethylated DNA, but which does not cleave unmethylated DNA. The parental, template DNA is almost always methylated or hemimethylated since most strains of *E. coli*, from which the template DNA is obtained, contain the required methylase activity. The remaining, annealed vector DNA incorporating the desired mutation(s) is transformed into *E. coli*.

In the design of a particular site directed mutagenesis experiment, it is generally desirable to first make a non-conservative substitution (e.g., Ala for Cys, His or Glu) and determine if activity is greatly impaired as a consequence. If the residue is by this means demonstrated to be important by activity impairment, or knockout, then conservative substitutions can be made, such as Asp for Glu to alter side chain length, Ser for Cys, or Arg for His. For hydrophobic segments, it is largely size that is usefully altered, although aromatics can also be substituted for alkyl side chains.

Other site directed mutagenesis techniques may also be employed with nucleic acid molecules encoding transcription factors useful in the practice of the present invention. For example, restriction endonuclease digestion of DNA followed by ligation may be used to generate deletion variants of transcription factors useful in the practice of the present invention, as described in Section 15.3 of Sambrook et al.

may be used to construct insertion variants, as described in section 15.3 of Sambrook et al., *supra*.

Oligonucleotide-directed mutagenesis may also be employed for preparing substitution variants of transcription factors useful in the practice of the present invention. It may also be used to conveniently prepare the deletion and insertion variants of transcription factors useful in the practice of the present invention. This technique is well known in the art as described by Adelman et al. (*DNA* **2**:183 [1983]); Sambrook et al., *supra*; "Current Protocols in Molecular Biology", 1991, Wiley (NY), F.T. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.D. Seidman, J.A. Smith and K. Struhl, eds., incorporated herein by reference.

Generally, oligonucleotides of at least 25 nucleotides in length are used to insert, delete or substitute two or more nucleotides in the nucleic acid molecules encoding transcription factors useful in the practice of the present invention. An optimal oligonucleotide will have 12 to 15 perfectly matched nucleotides on either side of the nucleotides coding for the mutation. To mutagenize wild-type transcription factor proteins useful in the practice of the present invention, the oligonucleotide is annealed to the single-stranded DNA template molecule under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of *E. coli* DNA polymerase I, is then added. This enzyme uses the oligonucleotide as a primer to complete the synthesis of the mutation-bearing strand of DNA. Thus, a heteroduplex molecule is formed such that one strand of DNA encodes the wild-type protein inserted in the vector, and the second strand of DNA encodes the mutated form of the protein inserted into the same vector. This heteroduplex molecule is then transformed into a suitable host cell.

Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA

simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions. The alternative method

involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type protein DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

Prokaryotes may be used as host cells for the initial cloning steps of inner ear cell transcription factors useful in the practice of the present invention. They are particularly useful for rapid production of large amounts of DNA, for production of single-stranded DNA templates used for site-directed mutagenesis, for screening many mutants and/or putative inner ear cell transcription factors simultaneously, and for DNA sequencing of the mutants generated. Suitable prokaryotic host cells include *E. coli* K12 strain 94 (ATCC No. 31,446), *E. coli* strain W3110 (ATCC No. 27,325) *E. coli* X1776 (ATCC No. 31,537), and *E. coli* B; however many other strains of *E. coli*, such as HB101, JM101, NM522, NM538, NM539, and many other species and genera of prokaryotes including bacilli such as *Bacillus subtilis*, other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may all be used as hosts. Prokaryotic host cells or other host cells with rigid cell walls are preferably transformed using the calcium chloride method as described in section 1.82 of Sambrook et al., *supra*. Alternatively, electroporation may be used for transformation of these cells. Prokaryote transformation techniques are set forth in Dower, W.J., in *Genetic Engineering, Principles and Methods*, **12**:275-296, Plenum Publishing Corp., 1990; Hanahan et al., *Meth. EnzyMol.*, **204**:63 (1991).

As will be apparent to those skilled in the art, any plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell may also be used to clone, express and/or manipulate nucleic acid molecules encoding inner ear cell transcription factors useful in the practice of the present [illegible] This [illegible] a replication site, marker genes that provide

[illegible] region containing several restriction sites [illegible] target DNA [illegible] plasmids

typically used for transformation of *E. coli* include pBR322, pUC18, pUC19, pUCI18, pUC119, and Bluescript M13, all of which are described in sections 1.12-1.20 of Sambrook et al., *supra*. However, many other suitable vectors are available as well. These vectors contain genes coding for ampicillin and/or tetracycline resistance which enables cells transformed with these vectors to grow in the presence of these antibiotics.

The promoters most commonly used in prokaryotic vectors include the β-lactamase (penicillinase) and lactose promoter systems (Chang et al. *Nature*, **375**:615 [1978]; Itakura et al., *Science*, **198**:1056 [1977]; Goeddel et al., *Nature*, **281**:544 [1979]) and a tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.*, **8**:4057 [1980]; EPO Appl. Publ. No. 36,776), and the alkaline phosphatase systems. While these are the most commonly used, other microbial promoters have been utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally into plasmid vectors (see Siebenlist et al., *Cell*, **20**:269 [1980]).

The construction of suitable vectors containing DNA encoding replication sequences, regulatory sequences, phenotypic selection genes and the DNA encoding a inner ear cell transcription factor useful in the practice of the present invention are prepared using standard recombinant DNA procedures. Isolated plasmids and DNA fragments are cleaved, tailored, and ligated together in a specific order to generate the desired vectors, as is well known in the art (see, for example, Sambrook et al., *supra*).

In the practice of the present invention, inner ear cells are genetically transformed with one or more nucleic acid molecules encoding one or more inner ear transcription factor proteins capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells). Any art-recognized gene delivery method can be used to introduce a nucleic acid molecule encoding an inner ear cell transcription factor into inner ear cells for expression therein. By way of non-limiting example, the following gene delivery methods can be used to introduce a nucleic acid molecule encoding an inner ear cell transcription factor into inner ear cells for expression therein: direct injection, electroporation, virus-mediated gene delivery, amino acid-mediated gene delivery, biolistic gene delivery and heat shock. Non-viral methods of gene delivery into inner

[illegible] incorporated herein by reference.

For example, genes can be introduced into cells *in situ*, or after removal of the cells from the body, by means of viral vectors. For example, retroviruses are RNA viruses that have the ability to insert their genes into host cell chromosomes after infection. Retroviral vectors have been developed that lack the genes encoding viral proteins, but retain the ability to infect cells and insert their genes into the chromosomes of the target cell (A.D. Miller, *Hum. Gen. Ther.* **1**:5-14 (1990)). Retroviruses will only efficiently infect dividing cells, thus when retroviruses are used to introduce genes into cells that have been removed from the body, cell division is stimulated with growth-promoting media or specific factors. *In vivo* application of retroviruses has been achieved by administration of virus-producing cells directly into tumors. Virus particle released by the infected cell will infect adjacent tumor cells, hence only a relatively small percentage of cells in a tumor need be initially infected in order to ultimately introduce the targeted gene into most or all of the tumor cells. (K.W. Culver et al., *Science* **256**:1550-1552 (1992)).

Adenoviral vectors are designed to be administered directly to patients. Unlike retroviral vectors, adenoviral vectors do not integrate into the chromosome of the host cell. Instead, genes introduced into cells using adenoviral vectors are maintained in the nucleus as an extrachromosomal element (episome) that persists for a limited time period. Adenoviral vectors will infect dividing and non-dividing cells in many different tissues *in vivo* including airway epithelial cells, endothelial cells, hepatocytes and various tumors (B.C. Trapnell, *Adv Drug Del Rev.* **12**:185-199 (1993)).

Another viral vector is the herpes simplex virus, a large, double-stranded DNA virus that has been used in some initial applications to deliver therapeutic genes to neurons and could potentially be used to deliver therapeutic genes to some forms of brain cancer (D.S. Latchman, *Mol. Biotechnol.* **2**:179-95 (1994)). Recombinant forms of the vaccinia virus can accommodate large inserts and are generated by homologous recombination. To date, this vector has been used to deliver interleukins (ILs), such as human IL-1β and the costimulatory molecules B7-1 and B7-2 (G.R. Peplinski et al., *Ann. Surg. Oncol.* **2**:151-9 (1995); J.W. Hodge et al., *Cancer Res.* **54**:5552-55 (1994)).

Another approach to gene therapy involves the direct introduction of DNA

liposomes in which the DNA is associated with one or more lipids, such as DOTMA (1,2,-diolcyloxypropyl-3-trimethyl ammonium bromide) and DOPE (dioleoylphosphatidylethanolamine). Formulations with DOTMA have been shown to provide expression in pulmonary epithelial cells in animal models (K.L. Brigham et al., *Am. J. Med. Sci*, **298**:278-281 (1989); A.B. Canonico et al., *Am. J. Respir. Cell. Mol. Biol.* **10**:24-29 (1994)). Additionally, studies have demonstrated that intramuscular injection of plasmid DNA formulated with 5% PVP (50,000 kDa) increases the level of reporter gene expression in muscle as much as 200-fold over the levels found with injection of DNA in saline alone (R.J. Mumper et al., *Pharm. Res.* **13**:701-709 (1996); R.J. Mumper et al., *Proc. Intern. Symp. Cont. Rol. Bioac. Mater.* **22**:325-326 (1995)). Intramuscular administration of plasmid DNA results in gene expression that lasts for many months (J.A. Wolff et al., *Hum. Mol. Genet.* **1**:363-369 (1992); M. Manthorpe et al., *Hum. Gene Ther.* **4**:419-431 (1993); G. Ascadi et al., *New Biol.* **3**:71-81 (1991), D. Gal et al., *Lab. Invest.* **68**:18-25 (1993)).

Additionally, uptake and expression of DNA has also been observed after direct injection of plasmid into the thyroid (M. Sikes et al., *Hum. Gene Ther.* **5**:837-844 (1994)) and synovium (J. Yovandich et al., *Hum. Gene Ther.* **6**:603-610 (1995)). Lower levels of gene expression have been observed after interstitial injection into liver (M.A. Hickman et al., *Hum. Gene Ther.* **5**:1477-1483 (1994)), skin (E. Raz et al., *Proc. Natl. Acad. Sci.* **91**:9519-9523 (1994)), instillation into the airways (K.B. Meyer et al., *Gene Therapy* **2**:450-460 (1995)), application to the endothelium (G.D. Chapman et al., *Circulation Res.* **71**:27-33 (1992); R. Riessen et al., *Human Gene Therapy*, **4**:749-758 (1993)), and after intravenous administration (R.M. Conry et al., *Cancer Res.* **54**:1164-1168 (1994)).

Various devices have been developed for enhancing the availability of DNA to the target cell. A simple approach is to contact the target cell physically with catheters or implantable materials containing DNA (G.D. Chapman et al., *Circulation Res.* **71**:27-33 (1992)). Another approach is to utilize needle-free, jet injection devices which project a column of liquid directly into the target tissue under high pressure (P.A. Furth et al., *Anal Biochem.* **20**:365-368 (1992); (H.L. Vahlsing et al., *J. Immunol. Meth.* **175**:11-22 (1994); (F.D. Ledley et al., *Cell Biochem.* **18A**:226 (1994)).

[illegible] Once within the nucleus the DNA dissolves from the gold [illegible]

microparticle and can be expressed by the target cell. This method has been used effectively to transfer genes directly into the skin, liver and muscle (N.S. Yang et al., *Proc. Natl. Acad. Sci.* **87**:9568-9572 (1990); L. Cheng et al., *Proc. Natl. Acad. Sci. USA.* **90**:4455-4459 (1993); R.S. Williams et al., *Proc. Natl. Acad. Sci.* **88**:2726-2730 (1991)).

Another approach to targeted gene delivery is the use of molecular conjugates, which consist of protein or synthetic ligands to which a nucleic acid- or DNA-binding agent has been attached for the specific targeting of nucleic acids to cells (R.J. Cristiano et al., *Proc. Natl. Acad. Sci. USA* **90**:11548-52 (1993); B.A. Bunnell et al., *Somat. Call Mol. Genet.* **18**:559-69 (1992); M. Cotten et al., *Proc. Natl. Acad. Sci. USA* **89**:6094-98 (1992)). Once the DNA is coupled to the molecular conjugate, a protein-DNA complex results. This gene delivery system has been shown to be capable of targeted delivery to many cell types through the use of different ligands (R.J. Cristiano et al., *Proc. Natl. Acad. Sci. USA* **90**:11548-52 (1993)). For example, the vitamin folate has been used as a ligand to promote delivery of plasmid DNA into cells that overexpress the folate receptor (e.g., ovarian carcinoma cells) (S. Gottschalk et al., *Gene Ther.* 1:185-91 (1994)). The malaria circumsporozoite protein has been used for the liver-specific delivery of genes under conditions in which ASOR receptor expression on hepatocytes is low, such as in cirrhosis, diabetes, and hepatocellular carcinoma (Z. Ding et al., *J. Biol. Chem.* **270**:3667-76 (1995)). The overexpression of receptors for epidermal growth factor (EGF) on cancer cells has allowed for specific uptake of EGF/DNA complexes by lung cancer cells (R. Cristiano et al., *Cancer Gene Ther.* **3**:4-10 (1996)). The presently preferred gene delivery method is lipofection.

When the methods of the present invention are utilized *in vitro*, the whole inner ear, including the Organ of Corti, is preferably excised and cultured and manipulated in a culture vessel. Presently preferred embodiments of an apparatus that is useful for culturing inner ears *in vitro* are disclosed in U.S. Patent Serial No. 5,437,998; U.S. Patent Serial No. 5,702,941 and U.S. Patent Serial No. 5, 763,279, each of which is incorporated herein by reference.

In general, presently preferred embodiments of an apparatus for culturing inner ears include a gas permeable bioreactor comprising a tubular vessel with walls [illegible] comprised [illegible] gas permeable material and the remaining portion made

nonpermeable material. The gas permeable materials commonly available are opaque. Thus, using nonpermeable material for at least part of the bioreactor may provide an advantage in allowing visual inspection of the tubular vessel chamber.

The tubular vessel has closed ends, a substantially horizontal longitudinal central axis, and one or more vessel access ports. The vessel access ports provide access to the bioreactor for input of medium and cells, and for removal of old medium from the tubular vessel. This is easily done through the vessel access ports which are also referred to as valves or syringe ports. In the preferred embodiment, the vessel access ports are constructed of valves with syringe ports.

Preferably the vessel is rotatable about its horizontal longitudinal central axis. A preferred means for rotation is a motor assembly which sits on a mounting base and has means for attachment to the tubular vessel. The speed of rotation can be adjusted so that the inner ear within the tubular vessel is constantly in motion, but rotation of the tubular vessel should not be fast enough to cause significant turbulence in the aqueous medium within the tubular vessel.

If so desired, the use of gas permeable material in the construction of at least part of the tubular vessel wall permits O_2 to diffuse through the vessel walls and into the cell culture media in the vessel chamber. Correspondingly, CO_2 diffuses through the walls and out of the vessel. Thus, the use of gas permeable material in the construction of at least part of the tubular vessel wall typically overcomes the need for air injection into the bioreactor vessel. Air injection into the aqueous medium within the bioreactor vessel may be utilized, however, if additional oxygen is required to culture an inner ear. When an air pump is utilized to inject air into the aqueous medium, an air filter is also employed to protect the air pump valves from dirt.

An alternative embodiment of the bioreactor useful in the practice of the present invention is an annular vessel with walls that may be constructed at least partially of a gas permeable material. Annular is defined herein to include annular, toroidal and other substantially symmetrical ring-like shaped tubular vessels. The annular vessel has closed ends and a substantially horizontal longitudinal central axis.

In another embodiment, the bioreactor useful in the practice of the present invention comprises a tubular vessel constructed at least partially of a gas permeable material. The vessel has closed ends and a substantially horizontal longitudinal central axis around which it rotates. The vessel furthermore has two slidably interconnected

bioreactor has means for rotating the tubular vessel about its substantially horizontal longitudinal central axis. One or more vessel access ports are provided for transferring materials into and out of the vessel.

In situations where minimization of contamination is necessary (e.g., AIDS or human tissue research), disposability of the bioreactor useful in the practice of the present invention is a particular advantage. Moreover, the embodiment of the bioreactor with slidably interconnected members may be adjusted to provide the exact size bioreactor needed.

Presently preferred, commercially available bioreactors useful in the practice of the present invention for culturing fluid-filled sensory organs are known as the High Aspect Ratio Vessel (HARV™) and the Cylindrical Cell Culture Vessel (CCCV™) and are manufactured by Synthecon, Inc. (8054 El Rio, Houston, Texas).

In the practice of the present invention, Neuralbasal™ media from Gibco BRL (Gibco BRL media are produced by Life Technologies, Corporate Headquarters, Gaithersburg, MD), which requires the addition of B27 or N2 media supplement, is the presently preferred culture medium. Other culture media can be successfully used, however, to culture fluid-filled sensory organs in the practice of the present invention. Other suitable media include DME, BME and M-199 with fetal calf serum or horse serum. All of the foregoing media are sold by Gibco -BRL. When using Neuralbasal™ medium, N2 or B27 supplements play a more significant role when extended periods of culture (>96 hr) are attempted.

In another aspect, the present invention provides methods for ameliorating the symptoms of an inner ear disease. In a presently preferred embodiment, the methods for ameliorating the symptoms of an inner ear disease comprise introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of inner ear cells (preferably capable of stimulating the regeneration of sensory hair cells from supporting cells), into a portion of the inner ear (preferably the cochlea, semicircular canals and/or otolithic organs) under conditions that enable the uptake of the nucleic acid molecule by inner ear cells and expression of the transcription factor within the inner ear cells. Presently preferred inner ear cells suitable for treatment in accordance with this aspect of the present invention are inner ear supporting cells. A nucleic acid molecule that encodes an inner ear cell

nucleic acid molecule that encodes an inner ear cell transcription factor

cochlea is by cochleostomy, *i.e.*, by puncturing the cochlea and inserting a catheter through which nucleic acid molecules encoding an inner ear cell transcription factor can be introduced into the cochlea. A cochleostomy method is disclosed, for example, in Lalwani, A.K. et al., *Hearing Research* **114**:139-147 (1997), incorporated herein by reference.

Regeneration of sensory hair cells within the inner ear is desirable in treating subjects suffering from an inner ear disease caused, at least in part, by the degeneration and/or death of inner ear sensory hair cells. Examples of diseases that affect inner ear sensory hair cells include: tinnitus, Meniere's Disease, vertigo, dysequilibrium, labyrinthitis and vestibulitis deafness.

The following examples merely illustrate the best mode now contemplated for practicing the invention, but should not be construed to limit the invention.

EXAMPLE 1

Presently Preferred Protocol for Transfecting Cultured Mouse Organ of Corti Cells

The present Example describes presently preferred protocols for transfecting cultured mouse Organ of Corti cells, and for identifying transcription factors that are effective to promote regeneration of inner ear cells. Previous attempts to transfect the Organ of Corti focused on unlesioned apical turn cochlear explants obtained from postnatal day 7-10 mice grown for a total of 5 days *in vitro* (DIV). For this procedure, mice prior to explant were sedated with halothone anesthesia before being sacrificed. Cultures were grown on glass slide wells (Nunc) coated with a solution of Cell-tak (Collaborative Research) in 100 µl of culture media containing 10% heat-inactivated horse serum (Gibco). Eight different lipid combinations were tested from the InVitrogen Perfect Lipofection Kit (InVitrogen). A bacterial plasmid encoding a β-galactosidase reporter gene driven by a CMV immediate/early gene promoter (InVitrogen) was delivered over a 4 hr period after 2 DIV followed by a recovery period of 3 DIV. The cultures were aldehyde-fixed and assessed for β-galactosidase expression using x-gal histochemistry. X-gal labeling appeared in a subset of supporting cells in the Organ of Corti and in several extrasensory regions of the cochlear explant.

Given the labor involved in detecting β-galactosidase expression, the size of the β-galactosidase encoding construct (4.1 kbp) and the reduced compatibility of this technique with other desired immunocytochemical procedures, a presently preferred [illegible] requires a standard [illegible]

and this gene has been successfully transfected into sensory cells and neurons within the cochlea using an AAV vector system (Lalwani, A.K. et al., *Hear. Res.*, 114:139-147 (1997)). However, one complication of the AAV vector system is that it readily penetrated the central nervous system (CNS) and transfected the contralateral cochlea when injected unilaterally *in vivo*. Therefore, lipofection is a preferred method over viral mediated transfections.

Organ of Corti cultures will be established from P7-P10 Swiss Webster mice derived from a breeding colony. The cultured organs will be transfected using a variety of commercially available lipofection reagents (i.e., FuGENE Transfection Reagent, Boehringer-Mannheim). These efficiencies will be compared against the transfection efficiencies achieved by the InVitrogen Kit. The optimal lipofection reagent and the optimal lipid to DNA ratio (3:1, 6:1 or 9:1) will be determined by counting the number of GFP-positive cells within the organ of Corti along a 250 μM length taken at the middle of the explant. Cells will be visualized using a Nikon epifluorescent microscope equipped with a CCD digital camera that will output images directly into Adobe Photoshop on a Macintosh computer where cell counts can be performed.

Using the best lipofection protocol determined as described above, an aminoglycoside antibiotic lesion of the hair cells will be performed, followed by subsequent lipofection. Media containing 1 mM neomycin sulfate (Sigma) for 2 DIV will be administered to kill the hair cells (>90%). Unlike early neonatal cultures, the older neonate is more easily affected by this concentration of neomycin resulting in the loss of greater than 90% of hair cells as determined by calbindin immunoreactivity (a hair cell selective marker). The remaining supporting cells will be lipofected for 4 hr with a GFP encoding plasmid. Cultures will be rinsed and grown in fresh media for an additional 3-6 DIV for a total of 5-8 days in culture post-explant. Cultures will be aldehyde–fixed and GFP will be visualized directly under epifluorescence. Efficiencies will be determined using the same methods previously described.

Example 2

Excision and *In Vitro* Culture of Mouse Inner Ear

The inner ear of a mouse was excised in the following manner. Postnatal day 7-14 Swiss Webster mice were decapitated and their skulls immersed in 70% ethanol for 5 min to disinfect. Under sterile conditions, the skull was cut into halves along the

Aurora Road, Naperville, IL 60563). Using surgical forceps, the bony inner ear labyrinth was visualized and separated from the temporal bone. The overlying connective tissue, stapes bone, facial nerve and stapedial artery were removed. Using a fine forcep, a small hole about 2 mm in diameter was made through the apical turn of the lateral cochlear wall. This surgically created conduit, along with the patent oval and round windows of the cochlea, permit ready diffusion of the culture media into the fluid-filled inner ear.

Typically, an inner ear excised and prepared in the foregoing manner is transferred to the HARV™ or CCCV™ vessel which contains 50 or 55 ml of Neuralbasal™ Media supplemented with either N2 or B27 media supplement (both sold by Gibco BRL, Catalogue number 17504-036), 10 U/ml of penicillin and .25 μg/μl of fungizone. The B27 supplement is sold as a 50X concentrate which is used at a working concentration of 0.5X (*e.g.*, 550 μl of 50X B27 stock solution is added to 55 ml of Neuralbasal™ Media). The N2 supplement stock solution is 100X and is used at a working concentration of 1X (*e.g.*, 550 μl of 100X N2 stock solution is added to 55 ml of Neuralbasal™ Media). The vessel is then placed in a tissue culture incubator at 37°C and in a 95% air/5% CO_2 environment. The vessel is then rotated at 39 rpm for periods of 24-168 hr. 50% media changes are made every 48 hr. As few as 2 and as many as 12 inner ears have been successfully cultured in one vessel.

To lesion the inner ear sensory hair-cells, the inner ear is placed in Neuralbasal™/N2 or B27 media that contain 1 mM neomycin sulfate (Sigma, P.O. Box 14508, St. Louis, MO 63178) for 24-48 hr. After this culture period, the media is completely replaced with media devoid of neomycin.

Example 3

Culture Media

Table 1 shows the composition of Neuralbasal™ medium (1x) sold by Gibco. All concentrations are working concentrations, *i.e.*, the concentrations of the components in the medium in which the fluid-filled sensory organ is incubated.

Table I. Neuralbasal™ media composition

Component	mg/liter	μM
Inorganic salts		
[illegible]		

Component	mg/liter	μM
KCL	400	5,360
$MgCl_2$ (anhydrous)	77.3	812
NaCl	3,000	51,300
$NaHCO_3$	2,200	26,000
$NaH_2PO_4H_20$	125	900
D-glucose	4,500	25,000
Phenol Red	8.1	23
HEPES	2,600	10,000
Sodium Pyruvate	25	230
Amino Acids		
L-alanine	2.0	20
L-arginine HCL	84	400
L-asparagine H_20	0.83	5
L-cysteine	1.21	10
L-glutamate		
Glycine	30	400
L-histidine HCL H_20	42	200
L-isoleucine	105	800
L-lysine HCL	146	5
L-methionine	30	200
L-phenylalanine	66	400
L-proline	7.76	67
L-serine	42	400
L-threonine	95	800
L-trptophan	16	80
L-tyrosine	72	400
L-valine	94	800
D-Ca pantothenate	4	8
Choline chloride	4	28
Folic acid	4	8
[illegible]		
Niacinamide		

Component	mg/liter	μM
Pyridoxal HCL	4	20
Riboflavin	0.4	10
Thiamine HCL	4	10
Vitamin B12	0.34	0.2

The following antibiotics may be added to Neuralbasal™ medium. Fungizone reagent (amphotericin B, 0.25μg/ml, and sodium desoxycholate, 0.25μg/ml) which is sold by Gibco-BRL, Catalog number 17504-036. Penicillin G (10 units/ml) which is sold by Sigma, Catalog number P 3414. Neomycin sulfate (1 mM), sold by Sigma, Catalog number N 6386. Neuralbasal™ medium may also be supplemented with L-Glutamine (2mM).

Example 4

Assay for Sensory Epithelium Vitality During Long Term Culture

In the practice of the present invention, the microgravitational environment provided by the rotation of the culture vessel allows the sensory epithelium of the inner ear to be maintained for prolonged periods of culture (>168 hr.) without significant degradation or loss of the sensory hair-cells or non-sensory supporting-cells. Data demonstrating the continued vitality of the sensory hair cells during prolonged culture were obtained by labeling the sensory epithelia with a probe against F-actin (phalloidin-FITC) that labels the surfaces of sensory and non-sensory cells, and with a hair-cell specific antibody against calbindin, a calcium binding protein. Both labels were detected and photographed under epifluorescence microscopy.

Cross-sectional data indicated that the normal cytoarchitecture of the inner ear sensory epithelia are maintained. For example, the Organ of Corti has several fluid-filled spaces called the tunnel of Corti and spaces of Nuel that are necessary for normal auditory function. These spaces occur between hair-cells and supporting-cells and are maintained after prolonged periods of culture. In normal gravitational environments, (*i.e.*, when the inner ear is floated without rotating the culture vessel) the sensory epithelia begin to degenerate. Without rotation, within 24 hr. the hair-cells are either completely missing or appear to be undergoing various endstages of cell death. After 48 hr., the supporting-cells are completely missing, or are present

EXAMPLE 5

Overexpression of POU4F3 in Lesioned Organ of Corti Cultures

POU4F3 is a DNA binding transcription factor that is specifically expressed in the hair cells in the inner ear. Mutations in POU4F3 are known to cause developmental failures in mice, and hearing loss in both mice and humans. A construct encoding POU4F3 will be spliced into a GFP encoding plasmid. This plasmid also contains an internal ribosome entry site (IRES) that permits the translation of two open reading frames (i.e., POU4F3 and GFP) from one mRNA. The advantage of using such a construct is that GFP production will coincide with the production of POU4F3. Transfected GFP-positive cells will be tested directly to ascertain that they are expressing POU4F3. Detection of exogenous POU4F3 will be determined using a polyclonal antibody (Santa Cruz Biotechnology or Babco). In addition, double-labeled cells (i.e., GFP and POU4F3) will be analyzed to determine whether they also express hair cell selective markers such as calbindin, a calcium binding protein, using an anti-calbindin monoclonal antibody (Sigma; Chemicon).

Cultures from P7-P10 mice will be established and lesioned with 1 mM neomycin for 2 DIV. The media will be removed and the cultures lipofected with pIRES-GFP-POU4F3 for four hours and permitted to recover in fresh media for 3-6 DIV. Cultures will be aldehyde-fixed and processed for POU4F3 and calbindin immunocytochemistry. Cultures lipofected with only pIRES-GFP will serve as controls. The presence of a triple labeled cell (positive for GFP, POU4F3, and calbindin immunoreactivity) would indicate that POU4F3 is capable of promoting the adoption of a hair cell phenotype in the lesioned organ of Corti. Further determination of this phenotype will be corroborated with other immunologic hair cell markers, such as Myo7a.

EXAMPLE 6

Overexpressing POU4F3 in Cultures to Identify Genes Regulated by POU4F3

Comparative hybridization of arrayed cDNAs has been used in a large variety of experiments including the identification of novel muscle-specific transcripts (Pietu, G. et al., *Genome Res.*, 6:492-503 (1996)), the discovery of inflammatory disease-related genes (Heller, R.A. et al., *PNAS USA* 94:2150-2155 (1997)), the analysis of gene expression during tumor suppression in a human melanoma cell line (DeRisi, J.

[illegible] RNA from cochlear explant cultures [illegible]

be isolated and radiolabeled via incorporation of ^{32}P-dCTP using a reverse transcription. One organ culture contains approximately 10,000 cells and yields up to 2 micrograms of total RNA. Two micrograms of total RNA is sufficient for expression analysis by hybridization to one set of cDNA grids.

The detection limit and dynamic range of cDNA hybridization arrays using nylon membranes to array cDNA clones and radioactively labeled cDNA probes are the two most critical parameters governing the types of genes which may be identified using this technology. Transcript abundance in a typical eukaryotic cell can be categorized as follows: low (1-10 copies per cell), medium high (100-500 copies per cell), and high (several thousand copies per cell) (Bishop, J.O. et al., *Nature*, **250:**199-204 (1974)). Several groups have now reported the detection of 1 transcript in 20,000 (Pietu, G. et al., *Genome Res.*, 6:492-503 (1996); Nguyen, C. et al., *Genomics*, **29:**207-216 (1995)) using nylon membranes. Based on an estimate of 10^5 to 10^6 transcripts per average eukaryotic cell (Bishop, J.O. et al., *Nature*, **250:**199-204 (1974)), the membrane-based high density cDNA array hybridization can detect between 5 and 50 mRNA molecules in a cell. While low level changes in the expression of low copy transcripts are not reliably detectable by this system, changes in transcript levels can be picked up within the medium-high or the high copy categories with reproducible results.

The two major factors governing the accuracy of the reading for one particular cDNA on a membrane are the amount of cDNA spotted on the membrane and the hybridization of the probe. For initial experiments, a set of nylon membranes containing arrayed cDNA clones representing a variety of human transcripts will be purchased and used (Clonetech Inc., Atlas Human cDNA Expression array or Research Genetics Human GeneFilters). These membrane sets provide a limited number of target genes but robust protocols have been developed for obtaining reliable results. Additionally, the manufacturers of these membranes have developed reliable means of spotting a consistent amount of cDNA to each location on each membrane. One of the two membranes will be hybridized with radiolabeled cDNA from GFP/POU4F3 transfected, aminoglycoside lesioned organ of Corti cultures. The remaining membrane will be hybridized with cDNA from aminoglycoside-lesioned organ of Corti cultures that have been transfected with the reporter GFP plasmid only.

Transfection efficiency will be monitored by determining expression of GFP from the construct containing the POU4F3 gene. Transfection efficiencies of the construct containing these two genes will be compared by fluorescence microscopy. Organ cultures with similar transfection efficiencies will be used for harvesting RNA and subsequent differential expression analysis.

Each of these RNA/cDNA hybridization pairs will be repeated with four independent sets of RNA on four independent sets of filters. This repetition will allow for statistical evaluation of the standard deviation of the hybridization intensity between filter groups probed with ^{32}P-labeled RNA from the same transfected cell culture system. True positive up-regulated or down-regulated genes will provide a reproducible result over the set of four membrane pairs. Imaging of the hybridization pattern will be done using the PhosphorImager storage phosphor system (Molecular Dynamics). Nylon membrane images stored on the phosphor screens are transferred to a Macintosh computer using an image splitting, spot finding, and spot integration software package (Crazy Quant V1.3) available freely through the laboratory of Dr. Leroy Hood. This is a highly flexible analysis package that integrates the hybridization intensity at each spot and stores values in a tab-delimited text file together with the coordinates of the spot on the array. For the exact determination of an individual hybridization ratio, the hybridization intensity values are imported into a ClarisWorks Spreadsheet for most calculations.

An anticipated transfection efficiency of 10% will allow for the identification of genes whose expression levels are changed by 50-fold or more due to the influence of POU4F3. Performing this series of experiments in cell lines is an alternative method that will allow for the identification of genes whose expression level is changed by less than 50-fold. Currently, there are no well characterized cell lines available derived from organ of Corti supporting cells. However, the ND-7 neuronal cell line does express POU4F3 and is viable following overexpression of POU4F3 (Smith, M.D. et al. Bcl-2, *Journ. Biol. Chem.*, 273:16715-16722 (1998)). This cell line will be used to identify POU4F3 transcriptionally regulated genes.

The value of identifying genes regulated by the transcription factor POU4F3 (Brn-3c) is intrinsic to the role of POU4F3 in the hair cell. It has been clearly demonstrated that POU4F3 is required for proper hair cell development in mice and [illegible] POU4F3 [illegible] in humans. Another member

[illegible] transcription. [illegible] Bcl-2 in neuronal cells (Smith, M.D. et al. Bcl-2 [illegible]

273:16715-16722 (1998)). This finding may explain why neuronal cells expressing Brn-3a are capable of escaping apoptosis following removal of nerve growth factor (NGF). Through the use of hybridization arrays, similarly intriguing genes regulated by POU4F1 may be identified, which genes may themselves act as target molecules in the pursuit of a biological remedy for hearing loss.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for stimulating the regeneration of an inner ear cell comprising introducing a nucleic acid molecule that encodes an inner ear cell transcription factor, capable of stimulating the regeneration of an inner ear cell, into at least one inner ear cell under conditions that enable expression of said transcription factor in said inner ear cell.

2. The method of Claim 1 wherein said inner ear cell is a supporting cell.

3. The method of Claim 1 further comprising the step of regenerating an inner ear sensory hair cell from said inner ear cell expressing said transcription factor.

4. The method of Claim 1 wherein said inner ear cell transcription factor is a POU4F3 homologue.

5. The method of Claim 4 wherein said POU4F3 homologue is POU4F3.

6. The method of Claim 5 wherein said POU4F3 comprises the amino acid sequence set forth in SEQ ID NO:2.

7. The method of Claim 5 wherein said nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:1.

8. The method of Claim 1 wherein said nucleic acid molecule is introduced into said inner ear cell by a method selected from the group consisting of direct injection, electroporation, virus-mediated gene delivery, amino acid-mediated gene delivery, biolistic gene delivery, heat shock and lipofection.

9. The method of Claim 8 wherein said nucleic acid molecule is introduced into said inner ear cell by lipofection.

10. A method for ameliorating the symptoms of an inner ear disease comprising introducing a nucleic acid molecule that encodes an inner ear cell

molecule by inner ear cells and expression of said transcription factor within said inner ear cells.

11. The method of Claim 10 wherein said portion of the inner ear is the cochlea.

12. The method of Claim 10 wherein said portion of the inner ear is the semicircular canals.

13. The method of Claim 10 wherein said portion of the inner ear is the otolithic organs.

14. The method of Claim 10 wherein said inner ear cell transcription factor is a POU4F3 homologue.

15. The method of Claim 14 wherein said POU4F3 homologue is POU4F3.

16. The method of Claim 15 wherein said POU4F3 comprises the amino acid sequence set forth in SEQ ID NO:2.

17. The method of Claim 15 wherein said nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:1.

18. The method of Claim 10 wherein said nucleic acid molecule is introduced into said portion of the inner ear by injection.

19. The method of Claim 11 wherein said nucleic acid molecule is introduced into the cochlea by cochleostomy.

20. The method of Claim 10 wherein said inner ear cells are inner ear supporting cells.

21. The method of Claim 20 further comprising the step of regenerating nner ear sensory hair cells from said inner ear supporting cells expressing said transcription factor

23. The transformed inner ear cell of Claim 22 wherein said transformed inner ear cell is a supporting cell.

24. The transformed inner ear cell of Claim 22 wherein said transformed inner ear cell is a sensory hair cell.

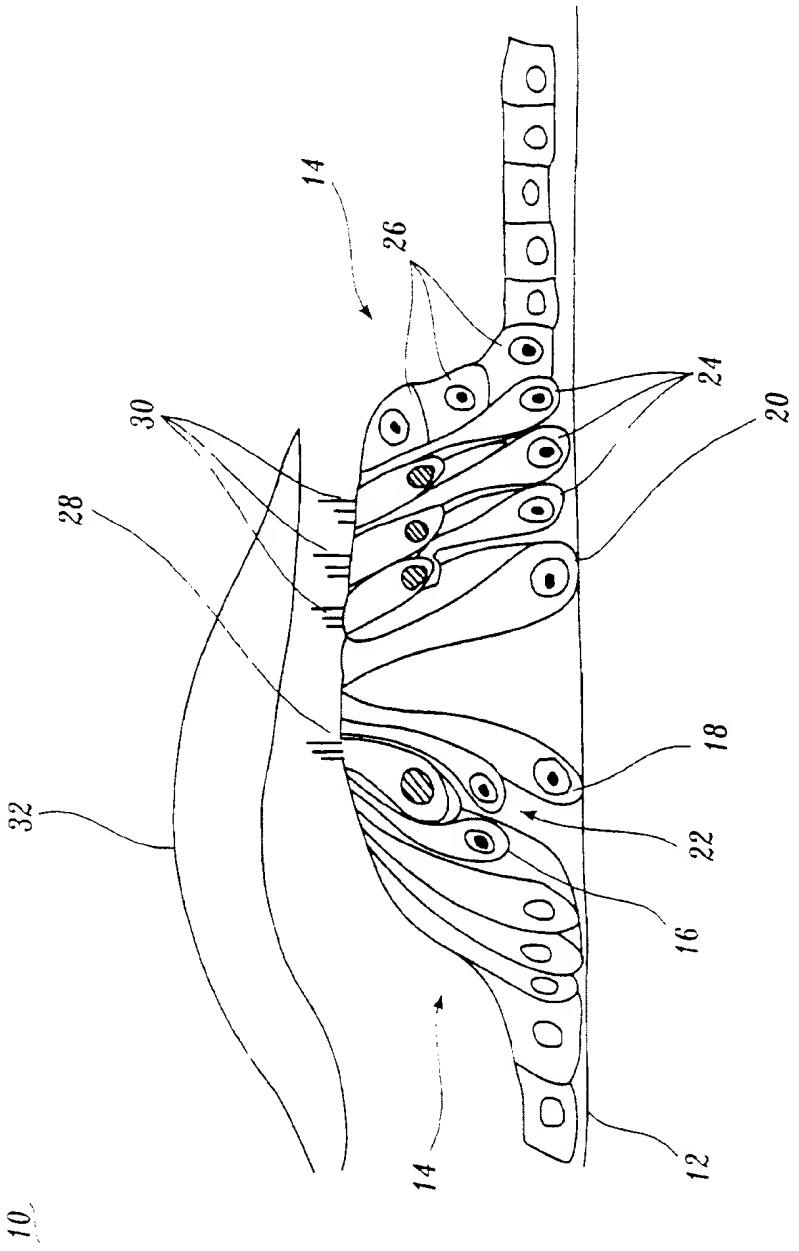
25. The transformed inner ear cell of Claim 22 wherein said inner ear cell transcription factor is a POU4F3 homologue.

26. The transformed inner ear cell of Claim 25 wherein said inner ear cell transcription factor is POU4F3.

27. The transformed inner ear cell of Claim 26 wherein said POU4F3 comprises the amino acid sequence set forth in SEQ ID NO:2.

28. The transformed inner ear cell of Claim 22 wherein said nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:1.

29. A method for stimulating the regeneration of an inner ear sensory hair cell from an inner ear supporting cell comprising introducing a nucleic acid molecule that encodes a POU4F3 homologue, capable of stimulating the regeneration of an inner ear cell, into at least one inner ear supporting cell under conditions that enable expression of said POU4F3 homologue in said inner supporting ear cell.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<120> METHODS FOR STIMULATING THE REGENERATION OF INNER EAR CELLS

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/24829

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70; A01N 43/04, 63/00, 65/00; C07H 21/02, 21/04

US CL :424 93.2; 514/44; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424.93.2; 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST; Chemical Astracts

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RYAN, A. F. Transcription Factors and the Control of Inner Ear Development. Seminars in Cell and Developmental Biology. 1997, Volume 8, pages 249-256, see especially pages 251-254.	1-29
Y	DE KOK, Y. J. M. et al. The Molecular Basis of X-Linked Deafness Type 3 (DFN3) in Two Sporadic Cases: Identification of a Somatic Mosaicism for a POU3F4 Missense Mutation. Human Mutation. 1997, Volume 10, 207-211, see especially pages 208-209.	1-29
Y	CREMERS, F. P. M. et al. Mapping and Cloning Hereditary Deafness Genes. Current Opinions in Genetics and Development. 1995, Volume 5, pages 371-375, especially pages 372-373.	1-29

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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11 FEB 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/24829

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VAHAVA, O. et al. Mutation in Transcription Factor POU4F3 Associated with Inherited Progressive Hearing Loss in Humans. Science. 20 March 1998, Volume 279, pages 1950-1954, see especially pages 1952-1953.	1-29
Y	GESCHWIND, M. D. et al. Defective HSV-1 Vector Expressing BDNF in Auditory Ganglia Elicits Neurite Outgrowth: Model for Treatment of Neuron Loss Following Cochlear Degeneration. Human Gene Therapy. 20 January 1996, Volume 7, pages 173-182, see pages 177-180.	1-29